Mutational Analysis of pre-mRNA Splicing in *Saccharomyces cerevisiae* Using a Sensitive New Reporter Gene, *CUP1*

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ABSTRACT

We have developed a new reporter gene fusion to monitor mRNA splicing in yeast. An intron-containing fragment from the *Saccharomyces cerevisiae ACT1* gene has been fused to *CUP1*, the yeast metallothionein homolog. *CUP1* is a nonessential gene that allows cells to grow in the presence of copper in a dosage-dependent manner. By inserting previously characterized intron mutations into the fusion construct, we have established that the efficiency of splicing correlates with the level of copper resistance of these strains. A highly sensitive assay for 5′ splice site usage was designed by engineering an *ACT1-CUP1* construct with duplicated 5′ splice sites; mutations were introduced into the upstream splice site in order to evaluate the roles of these highly conserved nucleotides in intron recognition. Almost all mutations in the intron portion of the 5′ consensus sequence abolish recognition of the mutated site, while mutations in the exon portion of the consensus sequence have variable affects on cleavage at the mutated site. Interestingly, mutations at intron position 4 demonstrate that this nucleotide plays a role in 5′ splice site recognition other than by base pairing with U1 snRNA. The use of *CUP1* as a reporter gene may be generally applicable for monitoring cellular processes in yeast.

THE goal of this work was to develop an improved genetic system by which to monitor pre-mRNA splicing *in vivo*. Intron-containing fragments of several *Saccharomyces cerevisiae* genes have previously been fused to reporter exons, including *β-galactosidase* (*LacZ*) from *Escherichia coli* (LARSON *et al.* 1983; TEEM and ROSBASH 1983), *HIS4* from *S. cerevesiae* (PARKER and GUTHRIE 1985) and *thymidine kinase* from the herpes simplex virus (FOUSER and FRIESEN 1986). These gene fusions have been used (1) to monitor the effects of intron mutations on the production of mature mRNA by measuring the activity of the fusion protein and (2) to genetically identify splicing components involved in intron recognition. To date, there have been only two reports of the successful use of the reporter genes for the genetic identification of *trans*-acting splicing components [*ACT1-HIS4* and *ACT1-LacZ* gene fusions (COUTO *et al.* 1987) and *CYH2-LacZ* gene fusions (NEWMAN and NORMAN 1991)].

The level of sensitivity and the range of detection of gene fusion reporter products are two very important criteria for the design of useful genetic schemes. *HIS4* gene fusions have limited genetic utility for both of these reasons. First, a high threshold concentration of HIS4 fusion protein needs to be produced in the cell before any growth on media containing histidinol (the biosynthetic precursor of histidine) can be detected (PARKER and GUTHRIE 1985; VIJAYRAGHAVAN *et al.* 1986). Second, once the threshold level is obtained, only a small range of increasing amounts of HIS4 fusion protein can be distinguished before the protein is no longer limiting for growth (VIJAYRAGHAVAN *et al.* 1986). In contrast, while *LacZ* fusions are very sensitive and provide relatively linear indicators of splicing efficiency, these reporters fail a third criterion: they cannot be used for the direct selection of suppressors; rather the β-galactosidase activity of each mutant candidate must be screened.

In order to avoid many of these limitations, we have designed a new gene fusion using *CUP1* as the reporter. *CUP1* is normally a nonessential gene (HAMER, THIELE and LEMONTT 1985), but it allows cells to grow in the presence of otherwise lethal concentrations of copper by chelating the metal in a dosage-dependent manner. *CUP1* has been demonstrated to provide a sensitive assay for studying gene amplification (WELCH *et al.* 1983; FOGEL *et al.* 1983). The threshold level of detectable CUP1 protein in a cell is very low and there is a large range (0.1–6.0 mM $CuSO_4$) over which different copper concentrations can be distinguished by monitoring growth on copper-containing media (FOGEL *et al.* 1983; HAMER, THIELE and LEMONTT 1985). In order to facilitate our studies of mRNA splicing, we constructed a gene fusion by combining the 5′ exon and intron-containing region of the yeast *ACT1* gene with the complete coding sequence of *CUP1*. In this way we are able to study the efficiency of pre-mRNA splicing by monitoring growth on copper-containing media.

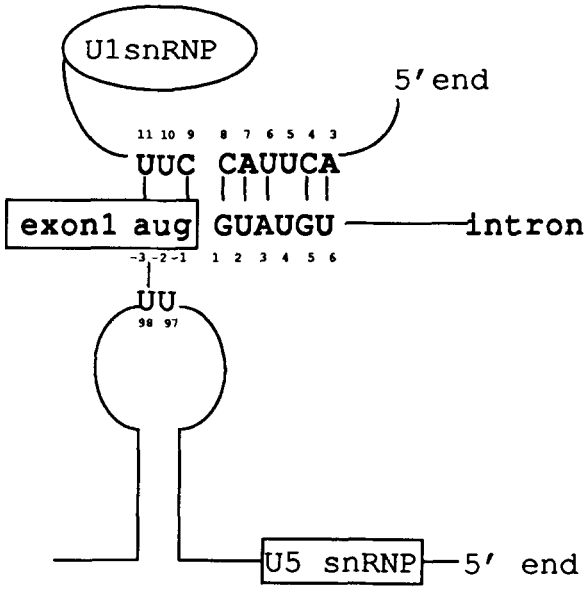

FIGURE 1.—Model of the proposed base-pairing interactions between the 5′ splice site and U1 snRNP and U5 snRNP (see text for references). The numbers beneath the 5′ss designate each of the positions of the consensus sequence.

We are interested in determining how introns are recognized. The only common components of all introns are three consensus sequences: the 5′ splice site (5′ss), branch point sequence and 3′ splice site (3′ss). Mutations in these consensus sequences demonstrate that they are important for both steps of splicing. In step 1, cleavage at the 5′ splice site results in the formation of free exon 1 and lariat intermediate. In step 2, ligation of free exon 1 to exon 2 results in the formation of mature mRNA and free intron in the lariat form [for review, see RYMOND and ROSBASH (1992), GUTHRIE (1991) and GREEN (1991)]. Our long-term goal is to employ the *ACT1-CUP1* fusion for the identification of *trans*-acting splicing factors involved in intron recognition. To evaluate the efficacy of this strategy, we have tested here a set of previously characterized intron mutations. We find, overall, an excellent correlation between growth on copper and the efficiency of splicing *in vivo*.

We have focused in particular on mutations in the 5′ splice site consensus sequence, since this region of the intron is known to have important roles in the earliest steps of spliceosome assembly. In yeast and mammals, the 5′ splice site is known to base pair with the 5′ end of U1 snRNA, [Figure 1; ZHUANG and WEINER (1986), SILICIANO and GUTHRIE (1988), SERAPHIN, KRETZNER and ROSBASH (1988)] and, in yeast, the U1 snRNP is the first snRNP to interact with the intron (RUBY and ABELSON 1988; SERAPHIN and ROSBASH 1989); if an intact branch point sequence is present, this interaction commits the intron to the splicing pathway (LEGRAIN, SERAPHIN and ROSBASH 1988; SERAPHIN and ROSBASH 1989). Several lines of evidence suggest that the consensus sequence at the 5′ splice site also plays other roles in addition to recognition by U1 snRNA. For example, recent genetic experiments in yeast have revealed an interaction between the exon portion of the 5′ splice site and U5 snRNA which can influence the precise location of 5′ splice site cleavage [Figure 1; NEWMAN and NORMAN (1991)]. By engineering duplicated 5′ splice sites in an *ACT1-CUP1* fusion, we have been able to probe the contribution of each nucleotide in the 5′ consensus region to 5′ splice site recognition. Interestingly, our results reveal a role for intron position 4 which does not involve base-pairing with U1 snRNA. Based on these findings, we conclude that the *CUP1* reporter gene fusion, particularly in the context of the splice site competition construct, should provide a sensitive system for the genetic identification of *trans*-acting components involved in splicing.

MATERIALS AND METHODS

Construction of the wild-type *ACT1-CUP1* plasmid: Site-directed mutagenesis techniques (KUNKEL, ROBERTS and ZAKOUR 1987) were used to introduce a *Sma*I restriction site at the ATG of *CUP1*, such that the ATG of *CUP1* is now GGG (pBS-Cup-Sma). This modification makes it possible to move all of the coding sequence and 167 nucleotides of the 3′-untranslated region of *CUP1* (KARIN *et al.* 1984) on a 353 nucleotide *Sma*I-*Acc*I fragment.

Site-directed mutagenesis techniques (KUNKEL, ROBERTS and ZAKOUR 1987) were next used to introduce a *Hpa*I site 11 nucleotides downstream of the *ACT1* 3′ splice site (NG and ABELSON 1980; GALLWITZ and SURES 1980). An *ACT1-CUP1* fusion was made by cloning the *CUP1* *Sma*I-*Acc*I fragment to the modified *ACT1* gene. The sequence at the **actin**-CUP1 junction is the following: **3′ss/AGGTTGCTGTT/** GGGTTCAGCGAA.

The gene fusion was then removed from the *ACT1* promoter and placed under the control of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter on the 2-μm pG1 plasmid [Figure 2; SCHENA, PICARD and YAMAMOTO (1991)]. Standard cloning techniques (SAMBROOK, FRITSCH and MANIATIS 1989) as well as polymerase chain reaction (PCR) methods (HIGUCHI 1989) were used to move each of the 5′ splice site mutations into the *Bam*HI or *Sal*I site of pG1. In addition, the selectable auxotrophy of pG1, *TRP1*, was replaced by *LEU2* in several clones. The following list specifies the name of the plasmid, the intron mutation, the selectable auxotrophy and the sequence of the junction region between *pG1* and **actin**. (Note: all restriction sites are underlined.)

pGAC1 (WT-*TRP1*): *GGATCGGTCGAC*CCAATTCGAGCTCGCC**CTTTT**.

pGAC20 (G5A-*LEU2*): *GGATCGGTCGAC*CAATT**CCTTTT**.

pGAC30 (G5T-*LEU2*): *GGATCC***CTTTT**.

pGAC40 (G5C-*LEU2*): *GGATCGGTCGAC*CAATT**CGCTTTT**.

pGAC60 (G1A-*LEU2*): *GGATCC***CTTTT**.

pGAC80 (G1C-*LEU2*): *GGATCGGTCGAC*CAATTCG**CTTTT**.

pGAC13 was constructed by making a fusion between pGAC1 and pG3 (SCHENA, PICARD and YAMAMOTO 1991). The italic sequence now represents *pG3* sequence.

pGAC13(WT-*TRP1*): *GGATCCCCGGGTACCGAGCTCG*-**CCCTTTT**.

pGAC13ΔKpn was made by removing the *Kpn*I site from pGAC13.

pGAC13ΔKpn(WT-*TRP1*): GGATCCCCGGCGAGCTCGCCCTTTT.

pGAC14 was made by introducing a *Kpn*I (*Asp*718) near the actin/**CUP1** junction so that the sequence there is: 3′ss/AGGTTGGTACC/**GGGTTCAGCGA**.

pGAC24 was made by replacing *TRP1* of pGAC14 with *LEU2*.

pGAC24(WT-*LEU2*): GGATCCCCGGCGAGCTCGCCCTTTT.

pGAC100(A259C-*LEU2*): the A259 mutation was introduced by site-directed mutagenesis techniques (KUNKEL, ROBERTS and ZAKOUR 1987) into pGAC24.

pGACΔ3′ss(*LEU2*) was introduced into pGAC24 by site-directed mutagenesis techniques. The junction sequence between *ACT1* and **CUP1** was altered to be the following: *TAC*AATATTCATCTC**CGAATTAA**.

The sequence at the 3′ junction of the gene fusion between *CUP1* and the **pG** vectors is the following in all of the clones: *TTATAGGTAT*GACCTGCAGCCCAAGCTG**GTCGAC**.

pM3: *prp16-1* (*TRP1*) is described in BURGESS, COUTO and GUTHRIE (1990).

pSE358: vector of pM3 (pSE358 was a gift from S. ELLEDGE).

Construction of the Δ*cup1* strain: The starting strain was ABDE1**a** Δ *cup1*: *MAT***a**, *arg4*, *CUP1Δ::URA3*, *thr1*, *his4*, *leu2*, *ade2*, *ade5*, *trp1*, *ura3*, *his7*. This strain was provided as a gift from the laboratory of S. FOGEL. ABDE1**a** was backcrossed three times to either TR2**a** or TR3α (gifts from the laboratory of P. HEITER). This strain was then mated to yPH399α (gift from P. HEITER). The resulting Δ*cup1* strains are:

K3 **a**Δ*cup1*: *MAT***a**, *cup1Δ::URA3*, *leu2*, *ura3*, *trp1*, *lys2*, *ade*, *GAL+*.

I4 αΔ *cup1*: *MAT*α *cup1Δ::URA3*, *leu2*, *ura3*, *trp1*, *lys2*, *ade*, *GAL+*.

Yeast transformations: Yeast lithium acetate transformations were done as described by BECKER and GUARENTE (1991).

Copper growth assays: Copper-resistant growth was determined by plate assays. Copper containing plates were made as described by SHERMAN (1991); however, Phytagar (GIBCO Laboratories) is substituted for Bacto-Agar (Difco). Plates were made to a chosen copper concentration by adding a dilution of filtered $CuSO_4$ (50 or 500 mM) solution to the agar solution after autoclaving and before pouring the plates. Copper was always added to complete synthetic media.

Copper-resistant growth was determined by either liquid spotting or replica plate assays. Liquid assays were done by growing up saturated overnights of the strain of interest in synthetic media which selects for the *ACT1-CUP1* plasmid. A 12.5-μl sample of each culture was then micropipetted onto a duplicate set of copper plates of numerous concentrations and growth was assayed after 3 days in order to determine the level of resistance of the strain. Replica plate assays were done as described by SHERMAN (1991). Copper-resistant levels were determined three days after replica plating.

RNA preparations: Cells were grown at 30° in 10 ml of the appropriate selective media to an OD_{600} of 0.8–1.2 and then harvested. RNA was isolated by a scaled down version of the method of WISE (1991). TNE (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA) was substituted for GTE buffer.

Primer extensions: Primer extensions were performed by the method of FRANK and GUTHRIE (1992). The exon 2 primer (3′Cup-CTTCATTTTGGAAGTTAATTAATT) used is complementary to *CUP1* and lies 11–34 from the *ACT1-CUP1* fusion junction. U1 snRNA was primer extended as an internal control for comparing the amount of RNA in each lane. The U1 snRNA primer used is (CAATGACTTCAATGAACAATTAT).

Mutagenesis of the upstream 5′ splice site: A 38-nucleotide oligonucleotide was synthesized (see Figure 6) which encodes two 5′splice sites separated by 13 nucleotides. A *Dra*I site is located between the two splice sites. The 5′ end of the oligonucleotide encodes a *Bgl*II site while the 3′ end of the oligonucleotide encodes a *Nhe*I site. The nine nucleotides of the upstream 5′ splice site were synthesized at a low level of degeneracy (97.8% correct, 2.2% mixture of the three incorrect nucleotides). The last six nucleotides of the oligo were designed as a palindrome, to facilitate conversion to a double-strand form through mutually primed synthesis (OLIPHANT, NUSSBAUM and STRUHL 1986). Hybridization and filling-in (OLIPHANT, NUSSBAUM and STRUHL 1986) yielded a pool of synthetic 5′ splice site competition fragments, each one bearing two 5′ss competitions and a central *Nhe*I restriction site. Cleavage with *Bgl*II and *Nhe*I resulted in individual 5′ss competition fragments each with a 5′ *Bgl*II end and a 3′ *Nhe*I end. 17% of the fragments theoretically should have a single mutation in the upstream 5′ss.

5′ Splice site competition plasmid construction: Site-directed mutagenesis techniques (KUNKEL, ROBERTS and ZAKOUR 1987) were used to introduce two restriction sites into pGAC14. A *Bgl*II site was introduced upstream of the 5′ss and a *Nhe*I site was introduced downstream of the 5′ss. A start codon (ATG) was also engineered just upstream of the *Bgl*II site.

This modified pGAC14 plasmid was digested with *Bgl*II and *Nhe*I and the 5′ss competition fragments were cloned into this vector. The ligation mixture was transformed into bacteria and a library was made by isolating plasmid DNA from 2200 pooled transformants. Since 159 colonies (27 [possible mutations]/0.17[probability of having a mutated 5′ss]) includes a complete genome of all possible mutations, the probability that the DNA library contains all possible mutations is 99.99% (probability $1 - e^{-n}$, where n = the number of genomes = 13.8).

Characterization of the wild-type competition: Plasmid DNA was isolated from 22 independent bacterial transformants. Ninety-one percent were of the correct restriction pattern. The wild-type 5′ss competition sequence was identified in 18/20 of the isolated plasmids. The wild-type competition plasmid was then transformed into the Δ*cup1* strain and analyzed by copper growth and primer extension assays.

The genetic copper selection for 5′splice site competition mutants: The DNA library was transformed into the Δ*cup1* strain. Several thousand yeast transformants were then replica plated onto 0.25 mM copper plates to select for mutants. Library DNA was isolated from approximately 120 transformants, which grew on 0.25 mM, copper by the bead-beating method of STRATHERN and HIGGINS (1991) and electroporated into bacteria by the method of DOWER, MILLER and RAGSDALE (1988). Plasmids were isolated from bacteria and checked by restriction digest to confirm that only one 5′ss competition fragment had been ligated to the vector (50% of the plasmids had the incorrect restriction pattern). Thirty plasmids which looked correct by restriction map were then sequenced (Table 1) by the method of SANGER, NICLEN and COULSON (1977) and retransformed into the Δ*cup1* strain. Transformants were then assayed for

TABLE 1

Summary of the number of specific mutations recovered at each position of the degenerate 5′ splice site

5′ splice site position	Mutation			
	G	A	T	C
−3	0	WT	0	0
−2	0	0	WT	0
−1	WT	0	0	0
1	WT	1	3	1
2	1	1	WT	1
3	1	WT	6	1
4	1	1	WT	3
5	WT	1	4	3
6	0	0	WT	0

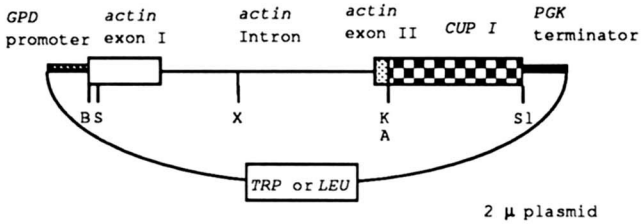

FIGURE 2.—Map of the *ACT1-CUP1* gene fusion. B, S, X, K, A and Sl refer to sites for restriction endonucleases *Bam*HI, *Sac*I, *Xho*I, *Kpn*I, *Asp*718 and *Sal*I, respectively.

copper-resistance levels. RNA was isolated from these strains and analyzed by primer extensions.

Changing the reading frame of the 5′ss competition plasmids: The 5′ss competition fragments were introduce into pGAC14, thus an *Asp*718 site lies downstream of the 5′ splice sites in the exon 2 coding sequence. When this restriction site is filled in, the mature RNA, generated as a result of cleavage at the upstream 5′ss, is now in-frame.

Site-directed mutagenesis: Intron position 6 and exon position −1 were made via *dut*⁻ *ung*⁻ mutagenesis techniques (KUNKEL, ROBERTS and ZAKOUR 1987). The exon position −2 mutants were made by PCR-oligo-directed mutagenesis (HIGUCHI 1989).

Quantitation of primer extension gels: All quantitation was phosphorimager done by scanning with a Molecular Dynamics phosphorimager. Final values were derived by averaging triplicate scans.

RESULTS

Construction of the *ACT1-CUP1* gene fusion: In order to monitor pre-mRNA splicing by assaying levels of copper-resistant growth, we cloned *CUP1*, the *S. cerevisiae* metallothionein homolog (WESER *et al.* 1977; KARIN *et al.* 1984), downstream from an intron-containing fragment of *ACT1*. To maximize the range over which to measure copper-resistant growth, the *ACT1* promoter was replaced by the strong constitutive GPD promoter and the gene fusion was placed on a high copy 2-μm vector (SCHENA, PICARD and YAMAMOTO 1991). Thus the fusion gene contains most of exon 1, all of the intron and 11 nucleotides of exon 2 of *ACT1* (NG and ABELSON 1980; GALLWITZ and SURES 1980) fused to the entire coding sequence

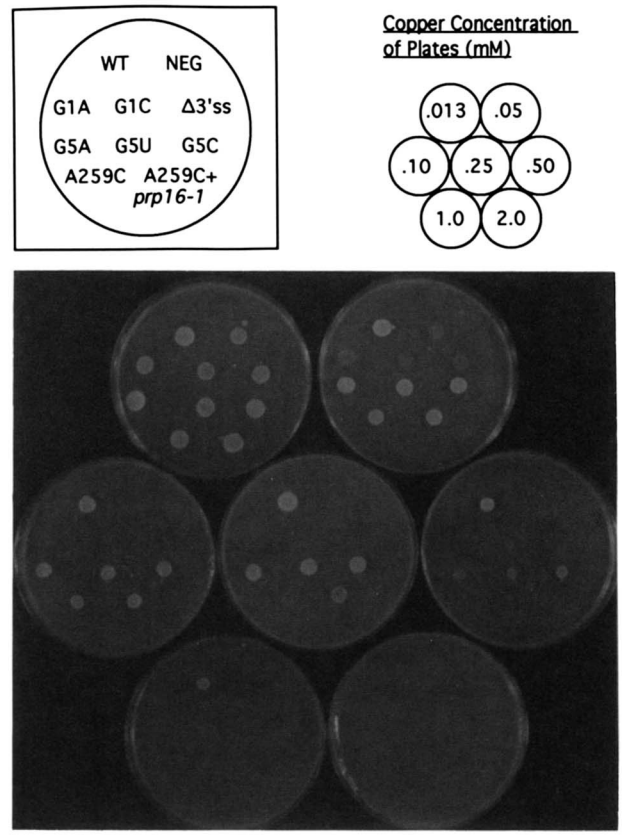

FIGURE 3.—Copper growth phenotypes of strains containing intron mutations. Strains containing each of the point mutations were assayed for copper-resistance levels by the liquid spotting method (see MATERIALS AND METHODS). The specific mutation carried by each Δ*cup1* strain is designated in the large circle. NEG indicates a strain carrying the vector of the *ACT1-CUP1* plasmid (pG1), but no *ACT1-CUP1* fusion gene. The specific copper concentrations of the plates are designated in the smaller circles.

of *CUP1* (Figure 2). The start codon of the gene fusion is that of *ACT1*, located ten nucleotides upstream of the intron.

A strain deleted for the chromosomal copy of *CUP1*, Δ*cup1*, is able to grow on media that lack copper, but fails to grow on media that contain >0.013 mM copper. Yeast transformed with the wild-type intron-containing gene fusion are able to grow on media that contain 1.0 mM copper (Figure 3). Thus, this system provides approximately a 100-fold range in which to measure copper-resistant growth.

Primer extension analyses with a primer complementary to the *CUP1* sequence of exon 2 allow us to quantitate the levels of precursor mRNA, lariat intermediate and mature mRNA produced. Primer extension analyses of RNA isolated from yeast containing the wild-type fusion gene demonstrate that the pre-mRNA can proceed through both steps of splicing (Figure 4A).

Characterization of intron mutations: In order to determine the relationship between splicing efficiency and levels of copper-resistant growth, previously characterized *ACT1* intron mutations were subcloned into

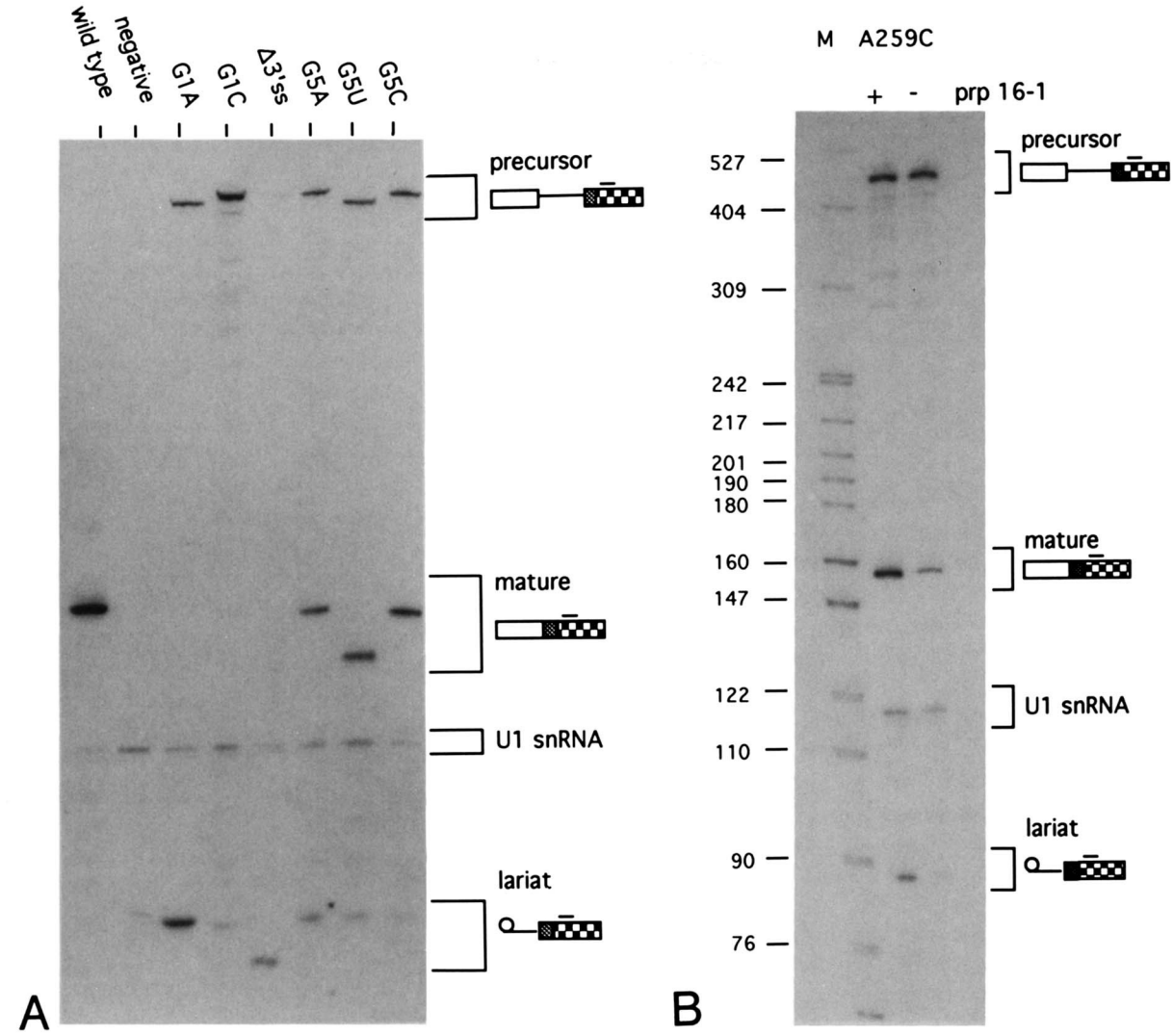

FIGURE 4.—Primer extension analysis of intron mutations. (A) Primer extension analysis of 5′ss and 3′ss mutations. RNA was prepared from strains carrying the intron mutation indicated at the top of each lane. Wild type indicates a strain transformed with an *ACT1-CUP1* construct containing the unmutagenized intron while negative indicates a strain that does not carry the *ACT1-CUP1* gene fusion. The sizes of the precursor and mature bands vary due to differences in the lengths of the 5′ untranslated regions resulting from variations in the cloning strategies for subcloning the intron mutations (see materials and methods). (B) Primer extension analysis of suppression of a branch point mutation. RNA was isolated from a strain carrying the A259C intron mutation and *prp16-1* or a control plasmid. The possible products of the primer extension reactions are diagrammed on the side of the gel. Each lane represents the products generated by primer extensions with *CUP1* and U1 snRNA oligos. The location of the exon 2 primer (3′Cup) is noted on the diagrams by a line above exon 2.

the fusion gene. Plasmids containing these intron mutations were transformed into the Δ*cup1* strain. Mutations were made in all three intron consensus sequences : the 5′ splice site (5′ss), branch point sequence and the 3′ splice site (3′ss).

Mutations in the 5′ss at intron position 1 (G1A and G1C), as well as mutations which delete the 3′ss (Δ3′ss), have previously been demonstrated to result in a block to the second step of splicing; mature product is not detected in these mutants (NEWMAN *et al.* 1985; VIJAYRAGHAVAN *et al.* 1986; FOUSER and FRIESEN 1986). Δ*cup1* strains containing *ACT1-CUP1* gene fusions with G1A, G1C or Δ3′ss mutations grew like strains which do not contain *ACT1-CUP1* gene fusions. That is, they were unable to grow on media which contain >0.013 mM copper (Figure 3, Table 2).

Primer extension analyses of RNA isolated from these strains demonstrate that G1A, G1C and Δ3′ss are unable to undergo the second step of splicing; they all accumulate lariat intermediate and produce no mature mRNA (Figure 4A). Mutations at intron position 1 also lead to the accumulation of precursor mRNA as a result of a decrease in the efficiency of step 1. As shown previously (VIJAYRAGHAVAN *et al.* 1986), this effect is much greater with G1C than G1A (Figure 4A).

All three intron position 5 mutations allow growth on copper plates, in the order: G5C > G5T ≥ G5A (Figure 3, Table 2). These mutations result in a de-

TABLE 2

Summary of copper growth and primer extension phenotypes of intron point mutations

Intron mutation	Copper growth (mM)	Mature/U1 SnRNA[a]
Wild type	1.0	32.5
No plasmid	0.013	0.0
G1A	0.013	0.0
G1C	0.013	0.0
Δ3′ss	0.013	0.0
G5A	0.30	2.4
G5U	0.30	3.4
G5C	0.50	6.9
A259C	0.15	1.6
A259C + *prp16-1*	0.25	2.7

[a] The ratio in this column indicates the amount of mature mRNA relative to the amount of U1 snRNA.

crease in the efficiency of both steps of splicing (PARKER and GUTHRIE 1985; JACQUIER, RODRIGUEZ and ROSBASH 1986; FOUSER and FRIESEN 1986; C. F. LESSER and C. GUTHRIE, unpublished). Primer extension analyses with an exon 2 primer demonstrate that precursor, lariat intermediate and mature products are present in each case (Figure 4A). Most importantly, when normalized to the amount of an internal control, the amount of mature product correlates with relative growth on copper plates: G5C > G5T ≥ G5A (Table 2). Thus, growth on copper-containing media accurately reflects the amount of spliced *ACT1-CUP1* mRNA.

The last intron mutation examined was the *ACT1* branch point mutation A259C, which has also been shown to result in a decrease in the efficiency of both steps of splicing (NEWMAN *et al.* 1985). Precursor, lariat intermediate and mRNA are observed by primer extension analyses (Figure 4B), demonstrating that the blocks to both steps of splicing are not complete. However, these strains produce less mRNA relative to an internal control compared to the strains transformed with intron position 5 mutations. Consistent with these results is the observation that Δ*cup1* strains containing this plasmid are less copper resistant than Δ*cup1* strains containing intron position 5 mutations (Figure 3, Table 2).

Genetic suppressor reconstruction experiment: The above results demonstrate that the phenotypes conferred by intron mutations introduced into the high copy *ACT1-CUP1* gene fusion closely reflect the phenotypes conferred by the same intron mutations on low copy *ACT1-HIS4* and *ACT1-LacZ* fusions. The increased sensitivity of the *ACT1-CUP1* system (see DISCUSSION) and the facility of the copper growth assays demonstrate that the *ACT1-CUP1* system is a quick and sensitive assay for characterizing *cis* mutations. To determine if the *ACT1-CUP1* system could also be used to find *trans*-acting suppressors of intron mutations, we asked whether a known unlinked suppressor of an intron mutation could suppress the same intron mutation in the *ACT1-CUP1* gene fusion.

prp16-1 is a dominant suppressor of the *ACT1* branch point mutation, A259C; *prp16-1* was isolated in a genetic selection for extragenic suppressors of the splicing defect conferred by the A259C mutation in an *ACT1-HIS4* gene fusion (COUTO *et al.* 1987). A plasmid containing the *prp16-1* allele was cotransformed with the A259C *ACT1-CUP1* gene fusion into the Δ*cup1* strain, which contains a wild-type chromosomal copy of *PRP16*. Yeast containing the *prp16-1* allele were able to grow on media containing 0.25 mM copper while yeast transformed with a control plasmid grew only on media containing 0.15 mM copper (Figure 3). Primer extension analyses also are consistent with suppression; approximately twofold more mature mRNA is produced in the presence of the second-site suppressor (Figure 4B, Table 2).

5′ Splice site competition assay: Next, we asked if this new gene fusion system could be used to assess the relative contributions of different nucleotides in the 5′ splice site region. An extremely powerful strategy for addressing the role of sequences in splicing has been to directly compare the activity of two sites in competition (REED and MANIATIS 1986). This type of competition assay can reveal contributions to splice site choice of components which affect this decision but are not normally rate-limiting for splicing. For example, a 3′ splice site competition assay was essential for demonstrating a role for a modestly conserved polypyrimidine tract in *S. cerevisiae* (PATTERSON and GUTHRIE 1991).

We thus established a 5′ splice site competition assay to examine the role of each of the nine nucleotides of the 5′ss consensus sequence. The 5′ splice site of *ACT1-CUP1* was replaced with a set of duplicated 5′ splice sites separated by 13 nucleotides (Figure 5A). In the first construct (reading frame I, Figure 5B), cleavage at the downstream 5′ splice site places the *ACT1* start codon in frame with the *CUP1* coding sequence. In a parallel construct (reading frame II, Figure 5C), only cleavage at the upstream splice site results in an in-frame fusion mRNA. Thus, by adjusting the reading frame it is possible to monitor use of either 5′ss by growth on copper-containing media.

When both sites in our competition construct are wild type, cleavage at the upstream site is favored. In reading frame I, when cleavage at the downstream site results in an in-frame mRNA, the ratio of the mRNAs generated by cleavage at the upstream site relative to the downstream site is only 1.7:1.0. However, if cleavage at the upstream site results in an in-frame mRNA, then the relative ratio is 6.6:1.0 (Figure 5D). Thus, the relative use of the upstream and downstream sites depends on which of the two mRNAs generated is in-frame, presumably because mRNAs

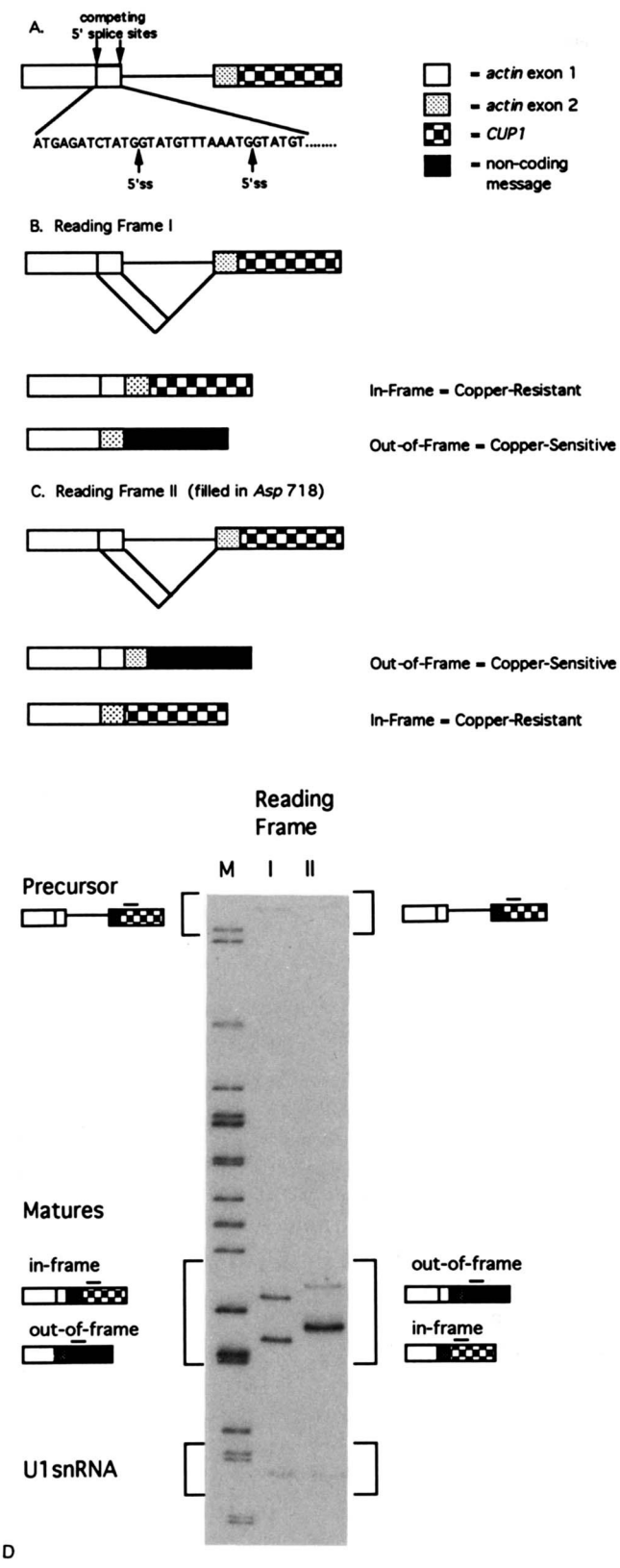

FIGURE 5.—5′ splice site competition assay. (A) General map of the 5′ss competition assay. The exact sequence of the 5′ss duplication is shown. The first ATG of the sequence is the new start codon of the fusion gene followed by the *Bgl*II restriction site and the first of the 5′ splice sites. The second 5′ss is located 13 nucleotides downstream from the first. (B) and (C) Schematic representations of the mature messages generated as a result of cleavage at each of the two possible 5′ splice sites. The relative reading frame of the competition constructs determines which of the two possible mature mRNAs will result in the production of a functional copper binding fusion protein. (D) Primer extension analysis of the reading frame I and reading frame II 5′ss competition constructs. RNA was isolated from a strain carrying the wild-type competition construct in either reading frame I or reading frame II. The possible products of the primer extension reactions are diagrammed on the side of the gel. Each lane represents the products generated by primer extensions with *CUP1* and U1 snRNA oligos. The location of the exon 2 primer (3′Cup) is noted on the diagrams by a line above exon 2. The difference in the sizes of the two sets of mature mRNAs is a result of filling in a restriction site to alter the reading frame.

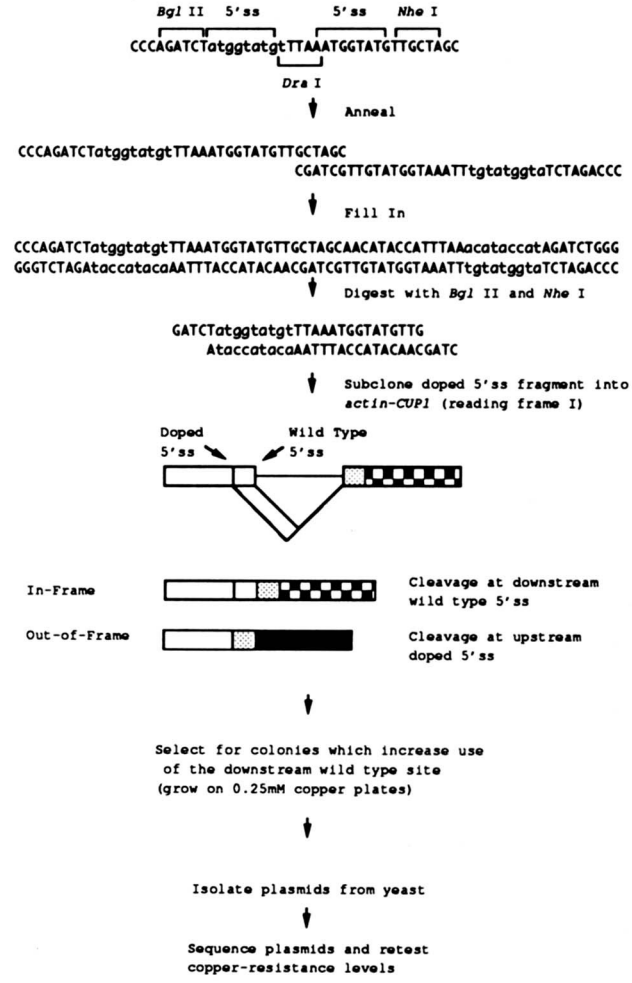

FIGURE 6.—Schematic representation of the strategy used to select for 5′ss mutations which affect the competition assay. (see MATERIALS AND METHODS for details.)

which contain stop codons near their 5′ ends have decreased stability (LOSSOM and LACROUTE 1979).

5′ Splice site competition mutagenesis: We created a bank of random mutations in the favored, upstream 5′ss by doped oligo-mutagenesis (Figure 6, see MATERIALS AND METHODS). In order to directly *select* for the mutations which affected 5′ss choice, we assumed that any mutation introduced into the highly con-

served upstream 5′ss that decreased or abolished use of that site would concomitantly increase use of the competing, unmutagenized downstream wild-type 5′ss. Therefore, the mutated 5′ss competition bank was cloned into reading frame I so that the level of copper resistance of strains carrying the mutant bank would reflect cleavage at the unmutagenized downstream site. Thus, we were able to isolate mutants by *selecting* for an increase in copper resistance rather than *screening* for a decrease in copper-resistance. Since the Δ*cup1* strain containing the unmutagenized reading frame I construct can not grow on media containing >0.18 mM copper, Δ*cup1* strains containing the mutagenized bank were screened for growth on media containing 0.25 mM copper. The DNA plasmids containing the mutagenized 5′ss competition were isolated from the strains and sequenced to identify the mutations.

As summarized in Table 1, all three possible nucleotide changes were isolated at intron positions 1, 2, 3, 4 and 5. No mutations were isolated at intron position 6 nor at the three exon nucleotides adjacent to the intron (positions −1, −2, −3). All 15 of the mutants at the first five intron positions can grow on some amount of copper >0.18 mM, but they vary in their upper tolerance (Table 3). In order to determine whether the differences in growth on copper media reflected the ability of different mutant 5′ splice sites to compete against the wild-type 5′ss, RNA was isolated from each of the mutants and analyzed by primer extension with the exon 2 *CUP1* primer. This primer is able to detect the two mature products generated by cleavage at each of the competing 5′ splice sites.

Primer extension analyses of mutants at positions 1, 2, 3 and 5 of the upstream site revealed no mRNA products which result from usage of the mutant site (data not shown). However, since cleavage at this mutated upstream 5′ss results in the production of an out-of-frame mRNA, the failure to detect such products may be due to the rapid turnover of the out-of-frame *ACT1-CUP1* mRNA. In contrast, all three changes at position 4 are able to compete with the wild-type site: wild type > T4C > T4G > T4A (Table 3).

To increase the sensitivity of the assay, the reading frame of all 15 point mutants was changed so that cleavage at the upstream mutagenized 5′ splice site would result in an in-frame (more stable) fusion mRNA. RNA was isolated from yeast transformed with each of these constructs and then analyzed by primer extensions with the exon 2 *CUP1* primer (Figure 7). Indeed, altering the reading frame made it possible to detect low level use of the mutant 5′ss in four cases: intron positions T2C, A3T, A3C and G5C. All three mutations at position 4 are still able to compete and in fact, they compete even better than before, presumably because cleavage now results in an in-frame, stable mRNA.

TABLE 3

Summary of copper-growth and primer extension phenotypes of 5′ss competition mutations

Intron mutation	Copper growth (mM)[a]	Wild-type mutant[b]
Wild type	0.18[c]	1.0/1.7[c]
T-2A	0.05	1.0/9.7
G-1T	0.18	1.0/1.6
G-1C	0.18	1.0/1.9
G1T	0.50	
G1A	0.50	
G1C	0.50	
T2G	0.50	
T2C	0.25	
T2A	0.50	
A3G	0.50	
A3T	0.50	
A3C	0.50	
T4G	0.25	1.0/.08
T4A	0.25	1.0/.06
T4C	0.50	1.0/.25
G5T	0.50	
G5A	0.50	
G5C	1.0	
T6A	0.013	
T6G	0.50	1.0/.26
T6C	0.50	1.0/.11

[a] Copper growth reflects the actin-CUP1 fusion protein produced as a result of cleavage at the downstream unmutagenized 5′ss.

[b] The ratios in this column indicate the amount of mature message generated as a result of cleavage at the unmutagenized downstream 5′ss relative to the mutagenized upstream 5′ss. Cleavage at the unmutagenized 5′ss results in an in-frame mRNA while cleavage at the mutant 5′ss results in an out-of-frame mRNA.

[c] Both of the 5′ splice sites in this competition are wild type.

Mutagenesis of intron position 6 and exon positions −2 and −1: Since no mutants were recovered at intron position 6 or in the three exon positions, we used site-directed mutagenesis to distinguish whether they have no effect in the competition or were simply missed in the original selection.

The last exon 1 position, G-1, was mutated to T (G-1T) and C (G-1C). These changes decrease the potential complementarity of the 5′splice site to the 5′ end of U1 snRNA (Figure 1). When use of the downstream site is assayed by copper growth, strains containing the mutant plasmids grow at the same copper levels as strains containing plasmids containing the wild-type 5′ss competition (Table 3), explaining why these mutants were not detected in our genetic selection. Similarly, neither mutant has an effect when assayed by primer extension (Figure 8).

Exon position −2 was mutated to an A (T-2A). This change increases the potential complementarity of the 5′ss to the regions of both U1 snRNA and U5 snRNA

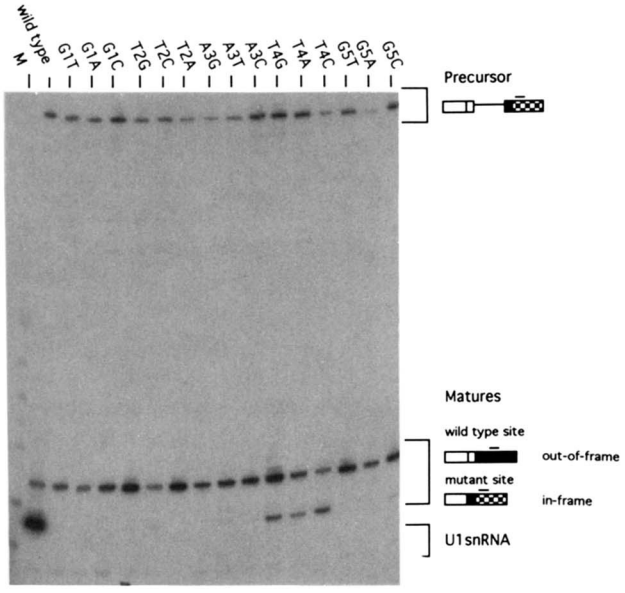

FIGURE 7.—Primer extension analysis of 5′ss competition mutations. RNA was prepared from strains carrying the 5′ss competition mutation indicated at the top of each lane. The 5′ss competition assays shown here are all in reading frame II. The possible products of the primer extension reactions are diagrammed on the side of the gel. Each lane represents the products generated by primer extension with *CUP1* and U1 snRNA primers. The location of the exon 2 primer (3′ Cup) is noted on the diagrams.

which have been demonstrated to base pair with the 5′ss [Figure 1; SILICIANO and GUTHRIE (1988), SERAPHIN, KRETZNER and ROSBASH (1988) and NEWMAN and NORMAN (1992)]. Primer extension analyses (Figure 8) and copper-resistance levels of strains containing this mutation (Table 3) demonstrate that the T to A mutation at this position actually increases cleavage at the mutated site relative to the wild-type site, resulting in a decrease in the level of copper-resistant growth. Consequently, it is not surprising that this change was not detected by the genetic selection scheme.

All three changes were made at intron position 6. When the wild-type T at position 6 is mutated to G (T6G) or C (T6C), primer extension analyses reveal that the mutated 5′ss can still compete with the wild-type 5′ss, although not as well as the wild-type site (Figure 8). Interestingly, these mutated 5′ splice sites compete better than changes at positions 1, 2, 3 and 5. When an A is introduced at this position (T6A), cleavage at the downstream wild-type site apparently results in the production of an unstable message due to the introduction of a stop codon (LOSSOM and LACROUTE 1979). When use of the downstream wild-type site is assayed by growth on media containing copper, the strains transformed with T6C and T6G can grow at 0.5 mM copper while yeast transformed with T6A grow at 0.013 mM copper. Thus mutants T6C and T6G should have been recovered in the genetic selection.

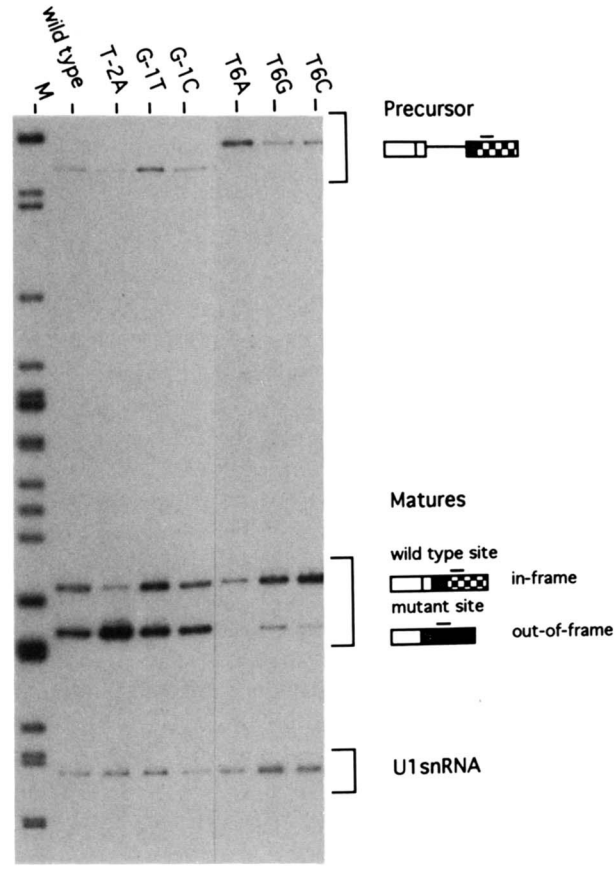

FIGURE 8.—Primer extension analysis of positions −2, −1 and 6 mutations. RNA was prepared from strains containing the 5′ss competition mutation indicated at the top of each lane. The 5′ss competition assays shown here are all in reading frame I. The products of the primer extension reactions are diagrammed on the side. Each lane represents the products of primer extension with CUP1 and U1 snRNA primers. The location of the exon 2 primer (3′ Cup) is noted on the diagrams.

DISCUSSION

Is the *ACT1-CUP1* gene fusion a good reporter to monitor pre-mRNA splicing? The intron-containing fragment of the yeast *ACT1* gene was fused to *CUP1* and the gene fusion was placed on a 2-μm vector under the control of the strong constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (SCHENA, PICARD and YAMAMOTO 1991). A careful analysis of the phenotypes conferred by a number of previously characterized *ACT1* intron mutants illustrates some of the advantages of the *ACT1-CUP1* system. First, it is extremely sensitive; that is, very low levels of the fusion protein can be detected by copper growth assays. This is in striking contrast to *ACT1-HIS4* fusions. For example, when the branch point mutation, A259C, is introduced into the *ACT1-HIS4* fusion gene, one cannot detect the production of mature mRNA by *in vivo* growth assays on media containing histidinol, although primer extension analyses demonstrate that mature mRNA is produced (VIJAYRAGHAVAN *et al.* 1986). However, a Δ*cup1*

strain containing an *ACT1-CUP1*-A259C fusion can grow on media that contains 0.18 mM copper, a concentration at least 10-fold greater than that at which the Δ*cup1* strain can grow. A second advantage to the *ACT1-CUP1* system, is the large range over which copper growth phenotypes can be monitored. For example, whereas the Δ*cup1* strain normally grows at 0.013 mM copper, the Δ*cup1* strain containing the wild-type *ACT1-CUP1* fusion grows at 1.0 mM copper. Therefore, the system provides about a 100-fold range in which the phenotypes conferred by different intron mutations can be compared. Third, the copper growth phenotypes of different intron mutants generally correlate with the amount of mRNA produced.

Finally, the *CUP1* gene fusion provides a new and powerful tool which can be used to design genetic selections and screens to identify *trans*-acting components. The sensitivity of the system allows the detection of even small changes in the copper growth phenotypes. It is possible either to directly select for changes which increase the ability of a strain to grow on copper-containing media or to screen for changes which decrease the ability of a strain to grow on copper-containing media. As just discussed, *CUP1* gene fusions are preferable to *HIS4* gene fusions because of their high sensitivity and large range of detection. In addition, *CUP1* gene fusions have an obvious advantage over *LacZ* gene fusions in that it is possible to directly select for improved growth rather than to screen for increases in β-galactosidase activity.

Analysis of the role of the nucleotides of the 5′ss in yeast introns; an application of *ACT1-CUP1*: A 5′ss competition assay was developed in yeast in order to further characterize the roles of the conserved nucleotides of the splice site consensus sequence. A second copy of the 5′ss was introduced 13 nucleotides upstream of the wild-type 5′ss of the *ACT1* gene. This *cis*-competition construct differs from that of GOGUEL *et al.* (1991) where the 5′ splice sites are separated by 75 nucleotides. In the latter case, cleavage occurs equally at each of the two 5′ splice sites. In our competition assay, however, although both of the 5′ splice sites are utilized, cleavage at the upstream site is significantly favored over the downstream site, 1.7–6.6-fold, depending on the relative reading frames of the two mRNAs. One explanation for this might be that in our construct the upstream site is adjacent to an authentic exon sequence while the downstream site is adjacent to a synthetic exon sequence; in the case of GOGUEL *et al.* (1991) both sites are flanked by authentic exon sequence. Splice site context has previously been demonstrated to affect splice site choice in mammalian 5′ss competition assays (REED and MANIATIS 1986; NELSON and GREEN 1988).

Mutagenesis of the upstream 5′ splice site: Single point mutations were introduced into each of the conserved nucleotides of the favored upstream 5′ss intron and exon sequences (ATG/GTATGT). A copper growth selection was then used to identify those mutations which reduce cleavage at the upstream site and thereby increase usage of the wild-type downstream site. In this selection, all three point mutation changes were recovered at intron positions 1, 2, 3, 4 and 5. Most changes at intron positions 1, 2, 3 and 5 resulted in complete loss of cleavage at the mutated site. These are the most highly conserved nucleotides of the consensus sequence (WOOLFORD 1989) and are complementary to the 5′ end of U1 snRNA. A base pairing interaction has been demonstrated between yeast U1 snRNA and intron positions 1 and 5 (SILICIANO and GUTHRIE 1988; SERAPHIN, KRETZNER and ROSBASH 1988). The most likely explanation for the effects of the mutations at intron positions 1, 2, 3 and 5 is thus that these mutations decrease the ability of the 5′ss to stably bind to U1 snRNA. Consequently, when these splice sites are competing with a wild-type splice site, U1 snRNA binds more stably to the wild-type 5′ss resulting in the commitment of this 5′ss to the splicing pathway. This model is consistent with that derived from the results of mammalian 5′ss competition assays where 5′ss choice appears to be dependent on the overall complementarity of the splice site sequence to U1 snRNA (NELSON and GREEN 1990; LEAR *et al.* 1991).

Suboptimal 5′ splice site sequences; role in regulation of gene expression: A striking correlation is that the three changes, at positions 2 and 3, which retain the ability to compete with the wild-type splice site, albeit at low levels, are all found in wild-type yeast introns. One gene, *COX5B* (HODGE and CUMSKY 1989) deviates from the 5′ss consensus sequence at position 2 (/GT to/GC) and two genes, *MER2* [/GTAT to/GTTC (ENGEBRECHT, VOELKEL-MEIMAN and ROEDER 1991)] and *RPL32* [/GTAT to/GTCA (DABEVA, POST-BEITTENMILLER and WARNER 1986)], deviate at position 3. Interestingly, the two genes which contain the position 3 variants also deviate from the consensus sequence at position 4 and these genes are the only two yeast genes whose expression is known to be regulated at the level of splicing. These results thus make it clear that yeast, like mammals, utilize suboptimal splice site sequences to regulate gene expression.

Intron position 6 influences 5′splice site choice: Intron position 6 is not as highly conserved as intron positions 1, 2, 3 and 5 (WOOLFORD 1989) and single point mutations at this position do not confer a detectable phenotype in an otherwise wild-type intron (SERAPHIN and ROSBASH 1989). However, a base pairing interaction can be demonstrated between position 6 and U1 snRNA in the presence of a second intron mutation in the branch point consensus sequence

(SERAPHIN and ROSBASH 1989). Mutations at position 6 in the competition assay compete far better than mutations at positions 1, 2 and 5, though not as well as the wild-type 5′ss. This result is consistent with the model that the longer a U1 snRNP stays bound to a 5′ss, the greater is the chance that this 5′ss will be utilized. By this view, the absence of base pairing between position 6 and the U1 snRNA would normally not be a rate-limiting defect; however, when competing with a wild-type 5′ss, a role for this nucleotide is uncovered. These results provide strong confirmation of the power of *cis*-competition assays to reveal the role of sequences not normally rate-limiting for splicing.

A novel role for intron position 4: The 5′ end of the *S. cerevisiae* U1 snRNA and the mammalian U1 snRNA are identical, yet the 5′ss consensus sequences differ at position 4. In yeast this position is almost invariably unpaired (WOOLFORD 1989). If the main criterion for 5′ss recognition in yeast is the ability to maximally base pair with U1 snRNA, as it is in mammals (NELSON and GREEN 1990; LEAR *et al.* 1991), then a 5′ss with a position 4 T to A mutation would be expected to be favored over a wild-type site. Our results demonstrate that this is definitely not the case. All three point mutations at this position are able to compete with the wild-type site. In fact, the favored nucleotide at this position is the wild-type T and the mutation which competes the best is a C, another pyrimidine. Therefore, it is likely that intron position 4 is examined independently of the base pairing interaction between the 5′ss and the U1 snRNP. Alternatively, the presumed mismatch at position 4, generated when the 5′ss and U1 snRNP are base paired, may be important for reasons of helical geometry. In either case, our results indicate that, unlike mammals, the sole determinant of 5′ss choice in yeast is not simply maximal base pairing with U1 snRNA.

The role of exon nucleotides in 5′ splice site choice: No mutations at any of the three exon positions tested were recovered in the genetic selection. In yeast, the exon nucleotide at position −1 is a G in 53% of the 53 sequenced intron-containing genes (RYMOND and ROSBASH 1992). Our analyses of two mutations synthesized by site-directed mutagenesis of position −1 of the upstream 5′ss confirmed that mutations at this position confer no phenotype in the competition assay. In mammals, however, position −1 is very important since this G, along with the first invariant G at intron position 1, is thought to define the cleavage site through its base pairing interaction with the U1 snRNA (AEBI, HORNIG and WEISSMANN 1987). Our genetic result, together with the sequence information, leads to the conclusion that in yeast, unlike in mammals, complementarity to U1 snRNA at position −1 is not very important. Taken together, the intron position 4 and exon position −1 results indicate that in addition to complementarity to U1 snRNA, yeast must employ some novel means to determine 5′ splice site specificity.

Exon position −2 is the least conserved nucleotide of the consensus sequence. It is often an A (36%) or a T (26%) (RYMOND and ROSBASH 1992). In our competition construct this nucleotide is a T. When it is mutated to A, cleavage at the mutant 5′ splice site is increased when in competition with the wild-type splice site. An A at this position is complementary to U1 snRNA (see Figure 1). This result is in contrast with our data for position −1, since changes of this nucleotide which should increase complementarity to U1 snRNA do not affect 5′ss choice. However, the advantage of an A at position −2 may not be due to a base pairing role with U1 snRNA. Recent results demonstrate that exon positions −2 and −3 can base pair with U5 snRNA and that this interaction can be important in cleavage site choice (NEWMAN and NORMAN 1991). The T to A mutation provides potential complementarity of this 5′ss nucleotide to both U1 snRNA and U5 snRNA. Conceivably, the different effects of mutations at positions −1 and −2 reflect the importance of the complementarity between −2 and U5 snRNA in determining the cleavage site choice. An alternative explanation is that when complementarity is increased between U1 snRNA and position −2, the length of the *continuous* region of potential base-pairing between U1 snRNA and the 5′ss increases from 4 to 6 base pairs. In the case of position −1 mutations, the wild-type splice site can base pair at five adjacent positions while the mutated splice sites can only base pair at three adjacent positions.

Summary: The combined power of the 5′ss *cis*-competition assay and the sensitive *ACT1-CUP1* reporter has allowed the direct genetic selection of a large set of 5′ss mutants from a mutagenized bank. The competition strategy was essential in uncovering a role for intron positions 4 and 6 and exon position −2, since mutations at these positions have no detectable phenotypes on their own. These findings argue that the *CUP1* fusions will continue to be an extremely useful tool in our genetic analysis of splicing. Moreover, in principle, *CUP1* can be fused to any gene or regulatory region in order to study other basic cellular processes. Presently, *CUP1* gene fusions are being used to study mRNA stability (R. PARKER, personal communication), transcription (J. LEFSTIN and K. YAMAMOTO, personal communication) and protein translocation (D. NG and P. WALTER, personal communication).

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LITERATURE CITED

AEBI, M., H. HORNIG and C. WEISSMANN, 1987 5′ Cleavage site in eucaryotic pre-mRNA splicing is determined by the overall 5′ splice region, not by the conserved 5′ GU. Cell **50:** 237–246.

BECKER, D. M., and L. GUARENTE, 1991 High-efficiency transformation of yeast by electroporation, pp. 182–186 in *Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology*, edited by C. GUTHRIE and G. R. FINK. Academic Press, San Diego.

BURGESS, S., J. R. COUTO and C. GUTHRIE, 1990 A putative ATP binding protein influences the fidelity of branchpoint recognition in yeast splicing. Cell **60:** 705–717.

COUTO, J. R., J. TAMM, R. PARKER and C. GUTHRIE, 1987 A trans-acting suppressor restores splicing of a yeast intron with a branch point mutation. Genes Dev. **1:** 445–455.

DABEVA, M. D., M. A. POST-BEITTENMILLER and J. R. WARNER, 1986 Autogenous regulation of splicing of the transcript of a yeast ribosomal protein gene. Proc. Natl. Acad. Sci. USA **83:** 5854–5857.

DOWER, W. J., J. F. MILLER and C. W. RAGSDALE, 1988 High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:** 6127–6145.

ENGEBRECHT, J., K. VOELKEL-MEIMAN and G. S. ROEDER, 1991 Meiosis-specific RNA splicing in yeast. Cell **66:** 1257–1268.

FOGEL, S., J. WELCH, G. CATHALA and M. KARIN, 1983 Gene amplification in yeast: *CUP1* copy number regulates copper resistance. Curr. Genet. **7:** 1–9.

FOUSER, L. A., and J. D. FRIESEN, 1986 Mutations in a yeast intron demonstrate the importance of specific conserved nucleotides for the two stages of nuclear mRNA splicing. Cell **45:** 81–93.

FRANK, D., and C. GUTHRIE, 1992 An essential splicing factor, *SLU7*, mediates 3′ splice-site choice in yeast. Genes Dev. **6:** 2112–2124.

GALLWITZ, D., and I. SURES, 1980 Structure of a split yeast gene: complete nucleotide sequence of the actin gene in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **77:** 2546–2550.

GOGUEL, V., X. LIAO, B. RYMOND and M. ROSBASH, 1991 U1 snRNP can influence 3′-splice site selection as well as 5′-splice site selection. Genes Dev. **5:** 1430–1438.

GREEN, M. R., 1991 Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. Annu. Rev. Cell Biol. **7:** 559–599.

GUTHRIE, C., 1991 Messenger RNA splicing in yeast: clues to why the spliceosome is a ribonucleoprotein. Science **253:** 157–163.

HAMER, D., D. THIELE and J. LEMONTT, 1985 Function and autoregulation of yeast copperthionen. Science **228:** 685–690.

HAMER, D., D. THIELE and J. LEMONTT, 1985 Function and autoregulation of yeast copperthionen. Science **228:** 685–690.

HIGUCHI, R., 1989 Using PCR to engineer DNA, pp. 61–70 in *PCR Technology: Principles and Applications for DNA Amplification*, edited by H. A. ERLICH. Stockton Press, New York.

HODGE, M., and M. CUMSKY, 1989 Splicing of a yeast intron containing an unusual 5′ junction sequence. Mol. Cell. Biol. **9:** 2765–2770.

JACQUIER, A., J. R. RODRIGUEZ and M. ROSBASH, 1985 A quantitative analysis of the effects of 5′ junction and TACTAAC box mutants and mutant combinations on yeast mRNA splicing. Cell **43:** 423–430.

KARIN, M., R. NAJARIAN, A. HASLINGER, P. VALENZUELA, J. WELCH and S. FOGEL, 1984 Primary structure and transcription of an amplified genetic locus: the *CUP1* locus of yeast. Proc. Natl. Acad. Sci. **81:** 337–341.

KUNKEL, T. A., J. D. ROBERTS and R. A. ZAKOUR, 1987 Rapid and efficient site-specific mutagenesis without phenotypic selection, pp. 367–382 In *Methods Enzymology, Recombinant DNA* (Part D), edited by R. WU and L. GROSSMAN. Academic Press, San Diego.

Larson, G. P., K. Itakura, H. Ito and J. ROSSI, 1983 *Saccharomyces cerevisiae actin-Escherichia coli LacZ* gene fusions: synthetic-oligonucleotide-mediated deletion of the 309 base pair intervening sequence in the actin gene. Gene **22:** 31–39.

LEAR, A. L., L. P. EPERON, I. M. WHEATLEY and I. C. EPERON, 1991 Hierarchy for 5′ splice site preference determined *in vivo*. J. Mol. Biol. **211:** 103–115.

LEGRAIN, P., B. SERAPHIN and M. ROSBASH, 1988 Early commitment of yeast pre-mRNA to the spliceosome pathway. Mol. Cell. Biol. **8:** 3755–3760.

LOSSOM, R., and F. LACROUTE, 1979 Interference of nonsense mutations with eucaryotic messenger RNA stability. Proc. Natl. Acad. Sci. USA **76:** 5134–5137.

NELSON, K., and M. GREEN, 1988 Splice site selection and ribonucleoprotein complex assembly during *in vitro* pre-mRNA splicing. Genes Dev. **2:** 319–329.

NELSON, K., AND M. GREEN, 1990 Mechanism for cryptic splice site activation during pre-mRNA splicing. Proc. Natl. Acad. Sci. USA **87:** 6253–6257.

NEWMAN, A., and C. NORMAN, 1991 Mutations in yeast U5 snRNA alter the specificity of 5′ splice-site cleavage. Cell **65:** 115–123.

NEWMAN, A. J., R. LIN, S. CHENG and J. ABELSON, 1985 Molecular consequences of specific intron mutations on yeast mRNA splicing *in vivo* and *in vitro*. Cell **42:** 335–344.

NG, R., and J. ABELSON, 1980 Isolation and sequence of the gene for actin in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **77:** 3912–3916.

OLIPHANT, A. R., A. NUSSBAUM and K. STRUHL, 1986 Cloning of random-sequence oligonucleotides. Gene **44:** 177–183.

PARKER, R., and C. GUTHRIE, 1985 A point mutation in the conserved hexanucleotide of a yeast 5′ splice junction uncouples recognition, cleavage and ligation. Cell **41:** 107–118.

PATTERSON, B., and C. GUTHRIE, 1991 A U-rich tract enhances usage of an alternative 3′ splice site in yeast. Cell **64:** 181–187.

REED, R., and T. MANIATIS, 1986 A role for exon sequences and splice-site proximity in splice-site selection. Cell **46:** 681–690.

RUBY, S. W., and J. ABELSON, 1988 An early hierarchic role of U1 small nuclear ribonucleoprotein in spliceosome assembly. Science **242:** 1028–1035.

RYMOND, B. C., and M. ROSBASH, 1992 YEAST PRE-MRNA SPLICING, IN *Molecular and Cellular Biology of the Yeast, Saccharomyces: Gene Expression*, edited by J. R. BROACH, J. R. PRINGLE and E. W. JONES. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (in press).

SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

SANGER, F. S., S. NICLEN and A. R. COULSON, *Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

SANGER, F. S., S. NICLEN and A. R. COULSON, 1977 DNA sequencing with chain-terminating inhibitors. Proc. Nat. Acad. of Sci. USA **74:** 5463–5467.

SCHENA, M., D. PICARD and K. R. YAMAMOTO, 1991 Vectors for constitutive and inducible gene expression in yeast, pp. 389–398 in *Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology*, edited by C. GUTHRIE and G. R. FINK. Academic Press, San Diego.

SERAPHIN, B., L. KRETZNER and M. ROSBASH, 1988 A U1 snRNA:pre-mRNA base pairing interaction is required early in yeast spliceosome assembly but does not uniquely define the 5′ cleavage site. EMBO J. **7:** 2533–2538.

SERAPHIN, B., and M. ROSBASH, 1989 Identification of functional U1 snRNA-pre-mRNA complexes committed to spliceosome assembly and splicing. Cell **59:** 349–358.

SERAPHIN, B., and M. ROSBASH, 1989 Mutational analysis of the

U1 small nuclear RNA and pre-mRNA of yeast. Gene **82:** 145–151.

SHERMAN, F., 1991 Getting started with yeast, pp. 3–20 in *Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology*, edited by C. GUTHRIE and G. R. FINK. Academic Press, San Diego.

SILICIANO, P. G., and C. GUTHRIE, 1988 5′ splice site selection in yeast: genetic alterations in base-pairing with U1 reveal additional requirements. Genes Dev. **2:** 1258–1267.

STRATHERN, J. N., and D. R. HIGGINS, 1991 Recovery of plasmids from yeast into *Escherichia coli*: shuttle vectors, pp. 319–328 in *Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology*, edited by C. GUTHRIE and G. R. FINK. Academic Press, San Diego.

TEEM, J. L., and M. ROSBASH, 1983 Expression of a β-galactosidase gene containing the ribosomal protein 51 intron is sensitive to the *rna2* mutation of yeast. Proc. Natl. Acad. Sci. USA **80:** 4403–4407.

VIJAYRAGHAVAN, U., R. PARKER, J. TAMM, Y. IIMURA, J. ROSSI, J. ABELSON and C. GUTHRIE, 1986 Mutations in conserved intron sequences affect multiple steps in the yeast splicing pathway, particularly assembly of the spliceosome. EMBO J. **5:** 1683–1695.

WELCH, J., S. FOGEL, G. CATHALA and M. KARIN, 1983 Industrial yeasts display tandem gene iteration at the *CUP1* region. Mol. Cell. Biol. **3:** 1353–1361.

WESER, U., H.-J. HARTMANN, A. FRETZDORFF and G.-J. STROBEL, 1977 Homologous copper(I)-(thiolate)$_2$ -chromophores in yeast copper thionen. Biochim. Biophys. Acta **493:** 465–477.

WISE, J., 1991 Preparation and analysis of low molecular weight RNAs and small ribonucleoproteins, pp. 405–414 in *Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology*, edited by C. GUTHRIE and G. R. FINK. Academic Press, San Diego.

WOOLFORD, J. L., 1989 Nuclear pre-mRNA splicing in yeast. Yeast **5:** 439–457.

ZHUANG, Y., and A. M. WEINER, 1986 A compensatory base change in U1 snRNA suppresses a 5′ splice site mutation. Cell **46:** 827–835.

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